

FORM PTO-1390  
(REV 5-93)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER  
1830/49264**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A  
FILING UNDER 35 U.S.C. 371**

U.S. APPLICATION NO (if known, see 37 CFR 1.5)

**09/647522**INTERNATIONAL APPLICATION NO.  
PCT/JP99/01607INTERNATIONAL FILING DATE  
March 30, 1999PRIORITY DATE CLAIMED  
April 1, 1998

## TITLE OF INVENTION

NOVEL HEMOLYTIC ACTIVE PROTEINS AND GENES ENCODING THE SAME

APPLICANT(S) FOR DO/EO/US  
Hiroshi NAGAI, Terumi NAKAJIMA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

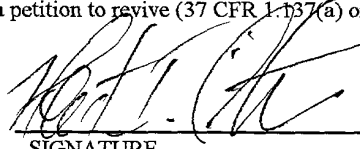
1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Item 11. to 16. below concern other document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.  
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

**23911**

PATENT TRADEMARK OFFICE

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) <b>09/647522</b>		INTERNATIONAL APPLICATION NO.		ATTORNEY'S DOCKET NUMBER 1830/49264	
17. <input checked="" type="checkbox"/> The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5)):				CALCULATIONS	
Search Report has been prepared by the EPO or JPO ..... \$860.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) .. \$690.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482)  but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ..... \$710.00 Neither international preliminary examination fee (37 CFR 1.482) nor  international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$10000.00 International preliminary examination fee paid to USPTO (37 CFR 1.482)  and all claims satisfied provisions of PCT Article 33(2)-(4) ..... \$100.00				PTO USE ONLY	
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				\$ 860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 130.00	
Claims	Number Filed	Number Extra	Rate		
Total Claims	20 - 20 =	0	X \$18.00	\$	
Independent Claims	2 - 3 =	0	X \$80.00	\$	
Multiple dependent claims(s) (if applicable)			+ \$270.00	\$	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$ 990.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$	
<b>SUBTOTAL =</b>				\$ 990.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
<b>TOTAL NATIONAL FEE =</b>				\$ 990.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$	
<b>TOTAL FEE ENCLOSED =</b>				\$ 990.00	
				Amount to be:	\$
				refunded	
				charged	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ 990.00 for the filing fee. b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees, which may be required, or credit any overpayment to Deposit Account No. <u>05-1323</u> . A duplicate copy of this sheet is enclosed. NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.437(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO: Evenson, McKeown, Edwards & Lenahan, P.L.L.C. 1200 G Street, N.W., Suite 700 Washington, D.C. 20005 Tel. No (202) 628-8800 Fax No. (202) 628-8844					
 SIGNATURE HERBERT I. CANTOR NAME 24,392 REGISTRATION NUMBER October 2, 2000 DATE					

09/647522

422 Rec'd PCT/PTO 0 2 OCT 2000

Attorney Docket: 1830/49264  
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: HIROSHI NAGAI ET AL.

§371 National Phase for PCT/JP99/01607

Serial No.: TO BE ASSIGNED      Group Art Unit: TO BE ASSIGNED

Filed: October 2, 2000      Examiner: TO BE ASSIGNED

Title: NOVEL HEMOLYTIC ACTIVE PROTEINS AND GENES  
ENCODING THE SAME

PRELIMINARY AMENDMENT

**Box PCT**

Commissioner for Patents  
Washington, D.C. 20231

Sir:

Prior to calculation of the fees and examination, please  
amend the above-identified application as follows:

IN THE SPECIFICATION:

Page 2,    line 2, change "cleared" to --found--;  
            line 3, change "same" to --some--;  
            line 14, before "non-dialyzable", insert --a--;  
            line 17, before "poison", insert --this--; and  
            line 24, change "have never been clarified up to  
now" to    --were not known up to now--.

Page 3,    line 19, change "clarified" to --found that--, and  
"having" to --had--;  
            line 24, after "...Ala-Thr", insert --(SEQ ID  
NO:1)--; and  
            line 27, after "...Val-Asn-Lys", insert --(SEQ ID  
NO:2)--.

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§371 National Phase for PCT/JP99/01607

Page 4, line 3, after "...Lys", insert --(SEQ ID NO:3)--;  
line 11, change "to" to --on the--;  
line 15, change "in" to --at the--; and  
line 21, change "(SEQ ID NO 5)" to --(SEQ ID  
NO:5)--.

Page 7, line 4, change "(SEQ ID NO 5)" to --(SEQ ID  
NO:5)--; and  
line 5, change "(SEQ ID NO 4)" to --(SEQ ID  
NO:4)--.

Page 8, line 1, change "(SEQ ID NO 4)" to --(SEQ ID  
NO:4)--.

Page 13, line 26, after "...Ala-Thr", insert --(SEQ ID  
NO:1)--.

Page 14, line 1, after "...Val-Asn-Lys", insert --(SEQ ID  
NO:2)--; and  
line 5, after "...Lys", insert --(SEQ ID NO:3)--.

Page 15, line 15, after "...CCI G", insert --(SEQ ID NO:6)-  
-;  
line 16, after "...TYT C", insert --(SEQ ID NO:7)-  
-;  
line 17, after "...AAY MG", insert --(SEQ ID  
NO:8)--;  
line 18, after "...ACI GC", insert --(SEQ ID  
NO:9)--;

line 19, after "...ATG G", insert --(SEQ ID NO:10)--;

line 20, after "...CRT C", insert --(SEQ ID NO:11)--;

line 21, after "...GAR AA", insert --(SEQ ID NO:12)--; and

line 22, after "...CCR TC", insert --(SEQ ID NO:13)--.

Page 17, line 28, after "...GCA GC", insert --(SEQ ID NO:14)--; and

line 29, after "...TCA TC", insert --(SEQ ID NO:15)--.

Page 18, line 1, after "...ATC CG", insert --(SEQ ID NO:16)--;

line 2, after "...TAA CG", insert --(SEQ ID NO:17)--; and

line 3, after "...TAC AC", insert --(SEQ ID NO:18)--.

Page 20, line 2, change "(SEQ ID NO 5)" to --(SEQ ID NO:5)-  
-;

line 3, change "(SEQ ID NO 4)" to --(SEQ ID NO:4)-  
-;

line 4, change "(SEQ ID NO 1)" to --(SEQ ID NO:1)-  
-;

line 5, change "(SEQ ID NO 2)" to --(SEQ ID NO:2)-  
-;

line 6, change "(SEQ ID NO 3)" to --(SEQ ID NO:3)--;

line 7, change both occurrences of "(SEQ ID NO 5)" to --(SEQ ID NO:5)--;

line 8, change "(SEQ ID NO 5)" to --(SEQ ID NO:5)--; and

line 10, change "(SEQ ID NO 4)" to --(SEQ ID NO:4)--.

**IN THE CLAIMS:**

Please cancel Claims 1-16 without prejudice or disclaimer of the subject matter therein, and substitute therefor, new Claims 17-36 as follows:

--17. An isolated protein comprising the amino acid residue sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:5, or any of the aforementioned sequences modified by an addition or deletion of one or more amino acid residues, and/or a substitution of one or more amino acid residues by another amino acid residue, wherein said protein has hemolytic activity.

18. The protein according to claim 17, wherein the protein is isolated from a nematocyst of *Carybdea rastonii*.

19. An isolated nucleic acid molecule encoding the protein according to claim 17.

20. The isolated nucleic acid molecule according to claim 19, wherein the molecule comprises the nucleic acid sequence of SEQ ID NO:4.

21. An isolated nucleic acid molecule which hybridizes with the nucleic acid molecule according to claim 19.

22. An isolated protein produced by expression of a polynucleotide sequence encoding the amino acid residue sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:5 or a polynucleotide which hybridizes with said polynucleotide sequence.

23. A vector comprising the nucleic acid molecule according to claim 19.

24. A vector comprising the nucleic acid molecule according to claim 21.

25. A host cell transfected/transformed by the vector according to claim 23.

26. A host cell transfected/transformed by the vector according to claim 24.

27. A process for making a vector which expresses the protein according to claim 17, comprising incorporating an isolated nucleic acid molecule encoding said protein into said vector in an operatively-linked relation with a promoter.

28. A process for making a protein having hemolytic activity comprising culturing the host cell according to claim 25, and recovering the protein from said host cell or culture solution.

29. A process for making a protein having hemolytic activity comprising culturing the host cell according to claim 26, and recovering the protein from said host cell or culture solution.

30. A process for isolating the protein according to claim 17, comprising ultrasonication of a nematocyst of *Carybdea rastonii* in phosphoric acid buffer solution, and extracting and purifying supernatant fluid after centrifugation by the ion exchange high performance liquid chromatography and gel filtration high performance liquid chromatography to isolate said protein.

31. The process according to claim 30, wherein the extraction and purification of said supernatant fluid is performed using a 10mM phosphoric acid buffer solution (pH 6.0) containing not less than 0.1 M NaCl at no more than 10°C.

32. A pharmaceutical composition comprising the protein according to claim 17, as an active component.

33. A method of stimulating platelet agglutination, comprising administering a platelet agglutination stimulating amount of the protein according to claim 17, thereby stimulating platelet agglutination.

34. An antibody specifically reactive with the protein according to claim 17.

35. A pharmaceutical composition comprising the antibody according to claim 34.



36. A pesticide comprising the protein according to claim 17, as an active component.

REMARKS

Entry of the amendments to the specification and claims before examination of the application is respectfully requested. These claims have been amended to remove multiple dependencies and to put the claims in better form for prosecution. These claims patentably define over the art of record.

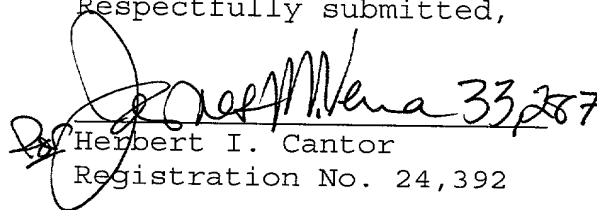
It is also respectfully requested that the above amendments be entered prior to calculation of the fees and prior to examination.

If there are any questions regarding this Preliminary Amendment or this application in general, a telephone call to the undersigned would be appreciated since this should expedite the prosecution of the application for all concerned.

If necessary to effect a timely response, this paper should be considered as a petition for an Extension of Time sufficient to effect a timely response, and please charge any deficiency in fees or credit any overpayments to Deposit Account No. 05-1323 (Docket #1830/49264).

Respectfully submitted,

October 2, 2000

 33,287  
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## SPECIFICATION

NOVEL HEMOLYTIC ACTIVE PROTEINS AND GENES ENCODING THE SAME

## 5 TECHNICAL FIELD

The present invention relates to proteins having a hemolytic activity and genes encoding thereof. More specifically, the present invention relates to novel proteins having the hemolytic activity, a process for producing and the  
10 use of the same.

## BACKGROUND ART

The sting injury by the jellyfish in sea bathing has occurred in various parts of the world. The sting injury by  
15 *Carybdea rastonii* or *Physalia physalis* has also occurred frequently in Japan every year in the season of sea bathing of the summertime. The degree of the symptom by sting differs by species of a jellyfish and the individual differences of patients. The first symptom is dermatoses, such as pain, flare,  
20 papule, vesicle and so on in the sting site. In a serious illness, patients may die with generating of hemorrhagic maculae and the necrosis, and also constitutional symptom, such as headache, high fever, nausea, dyspnea, and the fluctuation of a pulse. Although such sting injury is occurring frequently, the  
25 determination and pharmacological properties of the toxic components of jellyfish have not been studied intensively. Therefore, the development of medicines for treatment of the sting by the jellyfish is hardly performed before the present invention.

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The studies on the toxic components of *Carybdea rastonii* have reported by Sato et al., and they cleared that there are same active substances having physiological activities, such as hemolysis, platelet agglutination, mast cell degranulation, the vessel smoothness muscle contraction, the dermal necrosis, the heart poison and the fatality in the crude extract fractions from the freeze-dried tentacle of *Carybdea rastonii*. They also examined on the platelet agglutination effect and vessel smoothness muscle contraction effect of the toxic component (Akihiko Sato, "Research on the toxic component of *Carybdea rastonii*", *The Journal of the Ochanomizu Medico-dental Society*, vol. 33, No. 2, 131-151, June, 1985).

On the one hand, since the poison from the nematocyst of a jellyfish was non-dialyzable high polymer and deactivated by treatment with acid or alkali, or by heating processing, organic solvent processing, protease processing, etc., it was thought that the main components of poison were proteins.

Moreover, the purification of the protein toxin derived from a jellyfish has also been tried; however, the isolation and the purification of the active components maintaining the hemolytic activity were not performed since the toxin of a jellyfish itself was very easy to be deactivated. Therefore, the physical and chemical properties of the toxin from jellyfish have never been clarified up to now.

The detailed studies on the toxic component of a jellyfish is very important for the development of drugs applying their various physiological activities, in particular, specific hemolytic activity and the platelet agglutination effect.

Therefore, the problems to be solved by the present invention is providing an approach to development of the drugs for treatment of the sting injury by the jellyfish by means of isolating the proteins or peptides having as potent hemolytic activity as possible, in the state where the physiologic activity is retained. The present invention further provides the approach to study similarities on embryology or structure, and the species specificity of the protein having hemolytic activity to evaluate the structure-activity relationship thereof.

#### DISCLOSURE OF THE INVENTION

The inventors extensively performed the research for isolating the proteins having the hemolytic activity from the nematocyst of *Carybdea rastonii* using the hemolytic activity as the parameter, while retaining these hemolytic activities. As the result, they found out the process for isolating and purifying the proteins retaining hemolytic activities, and clarified the protein from *Carybdea rastonii* having the partial chemical structure consisting the following amino acid sequences (1)-(3), and the molecular weight of about 50,000 Da (determined by SDS gel electrophoresis).

Amino acid sequence (1):

Gly-Glu-Ile-Gln-Thr-Lys-Pro-Asp-Arg-Val-Gly-Gln-Ala-Thr

Amino acid sequence (2):

Gly-Asn-Ala-Glu-His-Val-Ala-Ser-Ala-Val-Glu-Asn-Ala-Asn-Arg-Val-Asn-Lys

Amino acid sequence (3):

Met-Ser-Asp-Gly-Phe-Tyr-Thr-Met-Glu-Asn-Ser-Asp-Arg-Arg-  
Lys

- 5 (wherein, an amino acid residue is written by the 3 letters notation defined by IUPAC and IUB)

10 Furthermore, they prepared the primers based on their partial chemical structures of the protein, and analyzed the gene sequence of about 1,000 base pair of said protein by conducting the RT-PCR to total RNA prepared from the tentacle of *Carybdea rastonii* by using these primers. Consequently, they further determined the full primary amino acid sequence of the hemolytic active protein of *Carybdea rastonii* by means of analyzing the gene sequence in 5'-end and 3'-end using the 5' RACE method and 3' RACE method.

20 Therefore, one embodiment of the present invention provides the specific protein having above-mentioned physiological, physical and chemical properties and represented by the amino acid SEQ ID NO 5, or the amino acid sequence thereof partially modified by the deletion or substitution of amino acid, and /or the amino acid sequence thereof partially modified by the deletion or substitution of amino acid further one or more amino acids are added.

Another embodiment of the present invention also provides the process for preparing such proteins.

Furthermore, another embodiment provides the gene encoding such proteins, the process for preparing the specific

proteins using the gene, and the drugs or the pesticides using the same.

The present invention further provides the pharmaceutical compositions or the pesticides containing the proteins using these properties, particularly, the pharmaceutical compositions having the platelet agglutination effect etc.

Moreover, since a specific antibody can also be obtained from this hemolytic active protein according to a conventional method (Cell Technology, separate volume, "*Experimental protocol of antipeptide antibody*", Shujunsha Co.), the present invention also provides the pharmaceutical compositions containing said antibody.

#### BEST MODE FOR CARRYING OUT THE INVENTION

The isolation and purification of the proteins having the specific physiological activity provided by the present invention can specifically be performed as follows. For example, the ultrasonication of the nematocyst of *Carybdea rastonii* is carried out in phosphoric acid buffer solution, and then supernatants are collected by the centrifugal separation to obtain a crude extract. The object proteins can be separated and purified by subjecting this crude extract to ion exchange high performance liquid chromatography using TSK-GEL (Toso Co.), and the gel filtration high performance liquid chromatography with Superdex-75 (Pharmacia Co.).

The structure of the protein provided according to the present invention obtained in this way can be determined by combining the analysis procedure of the amino acid sequence by the selective degradation using the enzyme, and the analysis

procedure of a gene sequence using the PCR method etc. For example, the amino acid sequence can be determined by processing the protein separated and purified as mentioned above with a lysylendopeptidase, fractionating the fragment using a high performance liquid chromatography, and analyzing it using an amino acid sequencer etc. Next, the gene sequence of the proteins can be determined by RT-PCR method etc. using the primers prepared on the basis of the amino acid sequence. Finally, the full primary amino acid sequence of the proteins can be clarified by determining the amino acid sequence on the basis of the gene sequence.

It was confirmed by such analysis that the protein provided according to the present invention has the molecular weight of about 50,000 Da (measured by SDS gel electrophoresis), and the partial amino acid sequences have the above-mentioned amino acid sequences (1) to (3).

As a result of homology search on the partial amino acid sequences, the homology between the protein of the present invention and the known proteins was very low. Therefore, it was suggested that the protein of the present invention having the hemolytic activity is completely novel protein, which is not similar to the known proteins.

Next, the determination of the gene sequence of about 1,000 base pairs by performing RT-PCR to total RNA prepared from the tentacle of *Carybdea rastonii* using the primers prepared on the basis of the partial amino acid sequence, and the determination of the gene sequences of the 5'-end and the 3'-end

using the 5' RACE method and 3' RACE method were performed. Consequently, it is concluded that the hemolytic active protein of *Carybdea rastonii* has the full primary amino acid sequence represented by SEQ ID NO 5, and the gene encoding thereof has the base sequence represented by SEQ ID NO 4.

The result of the homology search on these full primary amino acid sequences exhibited that the homology between the protein and the known proteins was low.

The method for preparing the specific protein of the present invention by separation and purification is characterized in retaining the hemolytic activity. For example, the separation and the purification in the state of retaining such hemolytic activity are attained by performing the processing such as ultrasonication using the above-mentioned phosphoric acid buffer solution or various high performance liquid chromatography in 10 mM phosphoric acid buffer solution (pH 6.0) containing above 0.1 M NaCl, preferably above 0.3 M, and more preferably above 0.5 M, at below 10°C, preferably below 5°C.

Therefore, the present invention also provides the method for preparing the protein by extracting and purifying them from the nematocyst of the *Carybdea rastonii* in the state of retaining the physiological activity.

The specific protein of the present invention also can be prepared by the gene recombination method. Preparation by the gene recombination method can be performed according to a conventional method. For example, it can be obtained by preparing the vector integrated with the gene represented by



SEQ ID NO 4, transforming a host cell by the vector, incubating or growing the host cell, and isolating and purifying the proteins having hemolytic activity of interest from the host cell or culture solution.

5 Since the protein provided according to the present invention has a hemolytic activity, for example, it may be used for the medicaments having the platelet agglutination effect and for the reagents for research on a hemolysis. Furthermore, it provides the new approach for the development of drugs, such  
10 as a drug for treating the sting by the jellyfish, and development of pesticides, such as an insecticide, using the hemolytic activity.

#### EXAMPLES

15 The present invention will be described in detail with reference to the following examples; however, the present invention is not limited to the examples.

##### Example 1

##### 20 1) Extraction of the nematocyst of *Carybdea rastonii*

200 mg of the nematocyst of the *Carybdea rastonii* obtained on the Miura peninsula, Kanagawa, Japan and cryopreserved at -80°C was immersed in 8 ml of 10 mM phosphoric acid buffer solution (pH 6.0), and treated for 15 minutes by the ultrasonic  
25 wave (ultrasonic cleaner VS150, Iuchi Co.). The supernatant fluids were collected by centrifugal separation (3,000rpm, for 20 minutes). This operation was performed 3 times in total. Furthermore, the same extraction operation was repeated 3 times with 8 ml of 10 mM phosphoric acid buffer solutions (pH 6.0)

containing 1 M NaCl, and then all the supernatant fluids were collected. After the extraction operation, ion exchange HPLC (high performance liquid chromatography) of the following purification step was immediately performed.

5

2) The purification by ion exchange HPLC (column: TSK-GEL CM650S, column size: 20 x 220 mm)

The above-mentioned column was equilibrated with 10 mM phosphoric acid buffer solution (pH 6.0) containing 0.3 M NaCl.

10 After the equilibration, the supernatant fluids obtained by extraction in the operation of the above-mentioned 1) were combined and diluted with 10 mM phosphoric acid buffer solution (pH 6.0) to 4 times. The solution was loaded onto the above-mentioned column at a flow rate of the 3 ml/min. The  
15 column was washed with 100 ml of 10 mM phosphoric acid buffer solutions (pH 6.0) after the sample application. The elution was carried out by the 60 minutes gradient in 0 to 0.7 M NaCl concentration (in 10 mM phosphoric acid buffer solution: pH 6.0). Hemolytic activity was showed in many fractions eluting  
20 between 45 and 65 minutes after start of the gradient. In addition, hemolytic activity was examined about the hemolytic effect to sheep hemocytes (see the after-mentioned example 2).

3) The purification by ion exchange HPLC (column: TSK-GEL CM5PW, column size: 7.5 x 75 mm)

25

The above-mentioned column was well equilibrated with 10 mM phosphoric acid buffer solution (pH 6.0) containing 0.3 M NaCl. The hemolytic active fractions obtained by purifying operation of the above-mentioned 2) were diluted with 10 mM

phosphoric acid buffer solution (pH 6.0) to 4 times. The solution was loaded onto the above-mentioned column at the flow rate of 2 ml/min. The column was washed with 30 ml of 10 mM phosphoric acid buffer solutions (pH 6.0) after the sample application. After washing, the elution was performed by the 60 min gradient in 0 to 0.8 M NaCl concentration (in 10 mM phosphoric acid buffer solution: pH 6.0). Fractions having hemolytic activity were eluted between 25 and 35 minutes after start of the gradient, and each fraction was applied to SDS-PAGE.

The separating condition of the active component was verified, and the portions separated well were collected and used in the next step. On the contrary, the portions not separated were further performed by chromatography to complete the separation of the active component.

- 4) Concentration of the hemolytic active component by ion exchange HPLC (column: TSK-GEL CM5PW, column size: 7.5 x 75 mm)

The column was well equilibrated with 10 mM phosphoric acid buffer solution (pH 6.0) containing 0.3 M NaCl. The hemolytic active fractions obtained by purifying operation of above-mentioned 3) were diluted with 10 mM phosphoric acid buffer solution (pH 6.0) to 4 times. The solution was loaded onto the above-mentioned column at the flow rate of 2 ml/min. The column was washed with 30 ml of 10 mM phosphoric acid buffer solutions (pH 6.0) after the sample application. After washing, 10 mM phosphoric acid buffer solution (pH 6.0) containing 0.8 M NaCl was then rinsed and the sample adhered into the column was allowed to elute. In about 5 minutes after exchange of

the solvent, the portion of the hemolytic active component condensed and eluted at a stretch was collected.

5) The purification by gel filtration HPLC (column: Superdex-75,  
5 column size: 16 x 600 mm)

Every 0.5-1.0 ml of the sample condensed by ion exchange HPLC was applied to the above-mentioned column equilibrated with 10 mM phosphoric acid buffer solution (pH 6.0) containing 0.8 M NaCl, and allowed to elute at the flow rate of 1 ml/min.

10 Potent hemolytic activity was found out in the fraction eluting between 50 and 60 minutes after injection of the sample. After confirming the separating condition by SDS PAGE, the protein of the present invention, a hemolytic toxin, was separated by collecting the active fractions (about 1 µg).

#### 15 Example 2: Measurement of the hemolytic activity

Measurement of the hemolytic activity in each purification step in the above-mentioned Example 1 and measurement of the hemolytic activity of the protein of the  
20 present invention finally obtained were performed as follows.

##### 1) Method

Hemolytic activity was measured by hemolysis to a sheep erythrocyte. That is, every 200 µl of PBS(+) buffer solution containing 0.8% of sheep erythrocyte was put into the microwell  
25 plates of 96 wells (round bottom type). 10 µl of the solution dissolved the fraction obtained in each purification step of the above-mentioned Example 1 in 10 mM phosphoric acid buffer solution (pH 6.0) was added to the plate. It was allowed to stand at room temperature for 3 hours, and the hemolytic

condition of the sheep erythrocyte of each plate was observed. In addition, the presence or absence of the retention of the hemolytic activity was determined by whether the fraction obtained in each purification step exhibits a perfect hemolysis.

5

## 2) Results

2-1) The fraction obtained in each purification step of the above-mentioned Example 1 exhibited the perfect hemolysis to the sheep erythrocyte, and therefore, it became clear that it  
10 retains the hemolytic activity.

2-2) Moreover, the protein of the present invention having the hemolytic activity finally obtained by purification operation of the above-mentioned 5) in Example 1 caused the perfect  
15 hemolysis to the sheep erythrocyte in the concentration below 100 ng/ml (about 2 nM).

## Example 3: Determination of the molecular weight and the partial structure on the proteins

20 3-1) Determination of the molecular weight

The single band visualized by applying the protein of the present invention having the hemolytic activity obtained by purification operation of 5) in Example 1 to SDS gel electrophoresis (SDS-PAGE) according to the conventional  
25 method was compared with the protein molecular-weight marker (Pharmacia Co.). As the result, it was identified that the molecular weight of the protein of the present invention are about 50,000 Da.

### 3-2) Decomposition with the lysylendopeptidase

The protein was decomposed by adding 3 pM of *Achromobacter* Protease I (derived from *Achromobacter lyticus* M497-1: Takara Shuzo Co.) to 10 µg of protein according to the present invention having the hemolytic activity obtained by purification operation of the above-mentioned 5) in Example 1, and incubating in 10 mM of Tris-HCl buffer solution (pH 9.0) at 30°C for 20 hours. The protein digested with the enzyme was applied to the high performance liquid chromatography (column: Bakerbond wide pore ODS), and separated with the 60 min gradient in 10 to 62% of acetonitrile concentration (in water containing 0.1% of trifluoroacetic acid) at the flow rate of 0.7 ml/min. Consequently, three peptide fragments eluting respectively at a retention time 19, 23 and 27 minutes were obtained.

### 3-3) Determination of the amino acid sequence of each fragments by the amino acid sequencer

The amino acid sequence of three peptide fragments obtained as mentioned above was determined according to the conventional method using Shimadzu PSQ-1 protein sequencer (Shimadzu Co.).

As the result, three fragments have the following amino acid sequences (1) - (3), respectively:

#### Amino acid sequence (1):

Gly-Glu-Ile-Gln-Thr-Lys-Pro-Asp-Arg-Val-Gly-Gln-Ala-Thr

#### Amino acid sequence (2):

Gly-Asn-Ala-Glu-His-Val-Ala-Ser-Ala-Val-Glu-Asn-Ala-Asn-

Arg-Val-Asn-Lys

Amino acid sequence (3):

Met-Ser-Asp-Gly-Phe-Tyr-Thr-Met-Glu-Asn-Ser-Asp-Arg-Arg-  
5 Lys

(wherein, an amino acid residue is written by the 3 letters notation defined by IUPAC and IUB).

10 The homology search about each fragment with which the amino acid sequence was determined as mentioned above exhibited that the homology between these fragments and the known proteins was very low. Therefore, it was suggested that the specific protein of the present invention fractionated from the  
15 nematocyst of *Carybdea rastonii* while retaining the hemolytic activity is completely novel protein.

Example 4: Determination of the full amino acid sequence of the protein and the gene encoding the amino acids

20 4-1) Preparation of total RNA of *Carybdea rastonii*

The tentacle (about 0.5 g in wet weights) of *Carybdea rastonii* was crushed in the liquid nitrogen, and homogenized in 5 ml TRIzol (registered trademark) reagent (GIBCO BRL Co.). To this mixture was added 1 ml of chloroform, and the mixture  
25 was agitated, and centrifuged with the cooling centrifuge (Sakuma Co.) [13,000rpm, for 15 minutes, at 4°C]. The upper aqueous layer was fractionated, and to this solution was added 2.5 ml of isopropanol, then, the mixture was allowed to stand at room temperature for 10 minutes. The supernatant fluid was

removed after the centrifugal separation (13,000rpm, for 10 minutes, at 4°C) using the cooling centrifuge, and then 5 ml of 75% ethanol was added the residue. The supernatant fluid was removed after the centrifuge (10,000rpm, for 5 minutes, at 4°C) to obtain the residue, then, the air-drying of the residue was performed for about 10 minutes. 100 µl of RNase-free water was added to the resulting residue, and the mixture was incubated for 10 minutes at 60°C to lyse RNA. About 0.5 mg of total RNA was obtained according to the above-mentioned method.

#### 4-2) Cloning of a partial cDNA

On the basis of amino acid sequence (1), amino acid sequence (2) and amino acid sequence (3), the following degenerate primers were designed and synthesized by the conventional method:

7-F; GAR ATH CAR ACI AAR CCI G

7-R; CIG GYT TIG TYT GDA TYT C

12-F; GCI GTI GAR AAY GCI AAY MG

12-R; CKR TTI GCR TTY TCI ACI GC

14-1-F; GAY GGI TTY TAY ACI ATG G

14-1-R; CCA TIG TRT ARA AIC CRT C

12-2-F; GAY GGI TTY TAY ACI ATG GAR AA

12-2-R; TTY TCC ATI GTR TAR AAI CCR TC

(wherein, the above-mentioned alphabetic character was written based on the "Nucleotide Abbreviation List" (Cell Technology, separate volume, *"Biotechnology Experiment Illustrated"*: Shujunsha Co.).

Next, according to the following procedure, single-strand cDNA was synthesized using SUPERSRIPT (registered trademark) Preamplification System for 1st-Strand cDNA Synthesis. That



is, 1 µg of total RNA , oligo(dT)<sub>12-18</sub>, and DEPC-treated water were mixed, and the mixture was allowed to stand for 10 minutes at 70°C. Then, PCR buffer, 25 mM MgCl<sub>2</sub>, 10 mM dNTP mix, and 0.1 M DTT were added to this mixture, and the resulting mixture was pre-incubated for 5 minutes at 42°C. Superscript II RT (200 units/µl) was added to this mixture, and the mixture was incubated for 50 minutes at 42°C and for 15 minutes at 70°C. The RNase H was added to the mixture, and then, the resulting mixture was incubated for 20 minutes at 37°C to obtain 1st-strand cDNA.

Subsequently, according to the following conditions, PCR was performed using GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer Co.). That is, 1st-strand cDNA, PCR buffer, dNTP mix, primer 1 and primer 2 (wherein, primer 1 and primer 2 are any eight above-mentioned primers.), TaKaRa Ex Taq (registered trademark, Takara Shuzo Co.), and water were mixed. The reaction was performed by heating the mixture at 94°C for 5 minutes and repeating 3 cycles of 30 seconds at 94°C, 30 seconds at 45°C and 2 minutes at 72°C, and 27 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 2 minutes at 72°C. The reactant was then treated for 5 minutes at 72°C.

The obtained reaction solution was electrophoresed on 0.8% agarose gel to confirm the amplified PCR products in the combination of 7-F and 12-R, 7-F and 14-1-R, 7-F and 14-2-R, 12-F and 14-1-R, and 12-F and 14-2-R. The sizes of each PCR product were about 600bp, 1,000bp, 1,000bp, 400bp, and 400bp, respectively.

#### 4-3) Sequencing of the partial cDNA

Each PCR product was inserted into TA cloning vector pCR2.1

094753.120103  
001021"22524350  
(Invitrogene Co.), and the recombinant was transformed to the *Escherichia coli* JM109. The transformant was cultured on LB (containing 50 µg/µl of ampicillin) agar medium. According to the following conditions, colony PCR was performed to the colonies obtained as a template using the M13 universal primer. The strain of *Escherichia coli*, PCR buffer, dNTP mix, M13 FW primer, M13 RV primer, TaKaRa Ex Taq (registered trademark, Takara Shuzo Co.), and water were mixed. The reaction was performed by heating the mixture at 90°C for 10 minutes and repeating 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 2 minutes at 72°C, and then heating at 72°C for 5 minutes. The reaction solution was electrophoresed on 0.8% agarose gel and the target colony PCR product was purified on the spin column of MicroSpin (registered trademark) S-400 (Amersham Pharmacia Co.). Then, the sequencing of the obtained product was conducted using ABI PRISM 310 Genetic Analyzer (Applied Biosystems Co.).

The obtained sequence was analyzed using gene analysis software GENETYX-MAC (Software Development Co.). As the result, the partial cDNA sequence of about 1000 bp was analyzed, and each partial structure of amino acid sequence (1), amino acid sequence (2) and amino acid sequence (3) was determined to locate in this turn from N terminal of the protein.

#### 4-4) Sequencing of the full-length cDNA

Following primers were synthesized based on the base sequence of the partial cDNA:

5'-RACE-4R; GCT CTA TCA ATA ACG GCA GC

5'-RACE-5R; TGT CTT TGG ATG GCC TCA TC

5'-RACE-6R; GAT ACT TAG GTC GCT ATC CG

3'-RACE-1F; GTT CAG AGG CTG TTC TAA CG

3'-RACE-2F; ATG TCT GAC GGC TTC TAC AC

Next, according to the following procedure, 5' RACE and  
5 3' RACE were performed using 5'/3' RACE Kit (Boehringer Mannheim  
Co.).

(a) 5' RACE

1 µg of total RNA, cDNA synthesis buffer, dNTP mix,  
5'-RACE-6R, AMV reverse transcriptase, and DEPC-treated water  
10 were mixed, and the mixture was incubated for 60 minutes at  
55°C and for 10 minutes at 65°C to obtain 1st-strand cDNA.

Next, 1st-strand cDNA thus obtained was purified on the  
spin column, then, reaction buffer and 2mM dATP were added to  
the 1st-strand cDNA, and the mixture was allowed to stand for  
15 3 minutes at 94°C. Terminal transferase (10 units/µl) was added  
to the mixture, and the resulting mixture was incubated for  
20 minutes at 37°C. After the incubation, 1st-strand cDNA, PCR  
buffer, dNTP mix, 5'-RACE-5R, oligo(dT)-anchor primer, and  
water were added to the above mixture. The reaction was  
20 performed by heating the mixture at 94°C for 5 minutes and  
repeating 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C  
and 1 minute at 72°C, and then heating at 72°C for 5 minutes.  
Consequently, the nested-PCR was performed to the 1st-PCR  
product as a template using the combination of 5'-RACE-4R and  
25 PCR anchor primer under the same condition as 1st-PCR.

The 1st-PCR product and the nested-PCR product were  
electrophoresed on 1.5% agarose gel to confirm the band of about  
500bp. This nested-PCR product was inserted into TA cloning  
vector, and the sequencing was performed according to the

determination of the base sequence of cDNA described in the above-mentioned 4-3), then the sequence was analyzed.

(b) 3' RACE

1 µg of total RNA, cDNA synthesis buffer, dNTP mix, oligo(dT)-anchor primer, AMV reverse transcriptase, and DEPC-treated water were mixed, and the mixture was incubated for 60 minutes at 55°C. Subsequently, the reactant was treated for 10 minutes at 65°C to obtain 1st-strand cDNA.

Next, 1st-PCR thus obtained was performed under the following condition. 1st-strand cDNA, PCR buffer, dNTP mix, 3'-RACE-1F, PCR anchor primer, TaKaRa Ex Taq (registered trademark, Takara Shuzo Co.), and water were mixed. The reaction was performed by heating the mixture at 94°C for 5 minutes and repeating 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 2 minutes at 72°C, and then heating at 72°C for 5 minutes. The nested-PCR was performed to the 1st-PCR product as a template using the combination of 3'-RACE-2F and PCR anchor primer under the same condition as 1st-PCR.

The 1st-PCR product and the nested-PCR product were electrophoresed on 1.5% agarose gel to confirm the band of about 600 bp. The nested-PCR product was inserted into TA cloning vector, the sequencing was performed according to the determination of the base sequence of cDNA described in the above-mentioned 4-3), and the sequence was analyzed.

As a result, the size (1610bp) and the sequence of cDNA encoding the novel hemolytic active protein of *Carybdea rastonii*, and the number (450aa) and the sequence of amino acid of the protein became clear. That is, the hemolytic active protein

of *Carybdea rastonii* had the amino acid sequence represented by SEQ ID NO 5, and the gene encoding thereof had the base sequence represented by SEQ ID NO 4.

The amino acid sequence (1) (SEQ ID NO 1), the amino acid sequence (2) (SEQ ID NO 2), and the amino acid sequence (3) (SEQ ID NO 3) corresponded to the amino acid number 56-69 of SEQ ID NO 5, the amino acid number 250-267 of SEQ ID NO 5, and the amino acid number 363-377 of SEQ ID NO 5, respectively. Furthermore, it was confirmed that the poly A sequence exists after the nucleotide number 1600 of SEQ ID NO 4.

The novel protein of the present invention obtained as mentioned above is the specific protein having the following physiological activity, and physical and chemical property, as indicated by the example:

- (a) having hemolytic activity;
- (b) having a molecular weight of about 50,000 Da (determined by SDS gel electrophoresis);
- (c) having the amino acid sequences 1 to 3 described above as a partial amino acid sequence; and
- (d) having the amino acid sequence represented by SEQ ID NO 5 as the full amino acid sequence.

#### Industrial applicability

Since the protein having the hemolytic activity derived from the nematocyst of *Carybdea rastonii* provided according to the present invention is a novel protein which is not similar to known protein, as a result of the homology search on the partial amino acid sequence and the full primary amino acid

sequences, it is useful as a biochemical reagent for example, elucidating the mechanism of a hemolysis etc.

5 It also provides the new approach directed to development of drugs, such as the medicine for treating the sting by the jellyfish, on the basis of study of correlation of the structural activity in a molecular level, and the antibody on the protein or the partial peptide, etc. Furthermore, it is useful as the drugs having a platelet agglutination effect etc., and pesticides using a hemolytic activity.

10

# SEQUENCE LISTING

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10 Phe Ile Val Leu Ala Ile Thr Ser Ala Lys His Gly Lys Arg Ser Asp

10

15

20

25

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30

35

40

15 ggt agc aac gag gct gct ctt gag gct tta gag ggc tta aaa gga gag 198

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45

50

55

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20

60

65

70

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75

80

85

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25

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90

95

100

105

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110

115

120



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5	gaa act agc aaa gca ata tta aat tac acg aaa tac ttt gga gtt cca	966		
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	300	305	310	
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20	ttg tat atc acc aag cat gac caa gga tgg gga tgg ggt act ttg gat	1206		
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[illegible]

440

1400

5

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1460

1520

1580

1610

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〈400〉 5

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10

15

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20

20

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30

Val Glu Thr Ala Leu Lys Glu Ala Ser Gly Ser Asn Glu Ala Ala Leu

35

40

45

Glu Ala Leu Glu Gly Leu Lys Gly Glu Ile Gln Thr Lys Pro Asp Arg

50

55

60

25

Val Gly Gln Ala Thr Lys Ile Leu Gly Ser Val Gly Ser Ala Leu Gly

65

70

75

80

Lys Leu Asn Ser Gly Asp Ala Thr Lys Ile Ile Ser Gly Cys Leu Asp

85

90

95

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	145	150	155	160
	Arg Asp Phe Ala Glu Ser Ser Ala Phe Ile Gln Val Met Lys Gln Gln			
	165	170	175	
10	Ser Asn Leu Thr Asp Ser Asp Leu Ser Ile Ile Ala Ala Asn Val Pro			
	180	185	190	
	Val Tyr Lys Phe Ser Asn Phe Ile Gly Gln Leu Glu Ser Arg Ile Ser			
	195	200	205	
	Gln Gly Ala Ala Thr Thr Ser Leu Ser Asp Ala Lys Arg Ala Val Asp			
15	210	215	220	
	Phe Ile Leu Leu Tyr Cys Gln Leu Val Val Met Arg Glu Thr Leu Leu			
	225	230	235	240
	Val Asp Leu Ala Ile Leu Tyr Arg Lys Gly Asn Ala Glu His Val Ala			
	245	250	255	
20	Ser Ala Val Glu Asn Ala Asn Arg Val Asn Lys Glu Leu Ala Ala Asp			
	260	265	270	
	Thr Leu Asp Phe Leu His Lys Leu Ile Pro Glu Gln Ala Leu Ile Gly			
	275	280	285	
	Ala Val Tyr His Pro Ile Ser Ala Ser Glu Thr Ser Lys Ala Ile Leu			
25	290	295	300	
	Asn Tyr Thr Lys Tyr Phe Gly Val Pro Asp Val Pro Arg Pro Ile Gly			
	305	310	315	320
	Asn Arg Arg Tyr Lys Phe Thr Asn Ser Tyr Trp Asn Thr Tyr Ser Ile			
	325	330	335	

Cys Ser Glu Ala Tyr Met Gly Asn Tyr Met Phe Arg Gly Cys Ser Asn

340

345

350

Val Arg Asn Pro Asn Ile Arg Val Ser Lys Met Ser Asp Gly Phe Tyr

355

360

365

5 Thr Met Glu Asn Ser Asp Arg Arg Lys Leu Tyr Ile Thr Lys His Asp

370

375

380

Gln Gly Trp Gly Trp Gly Thr Leu Asp Glu Asp Pro Gly Asp Gln Gly

385

390

395

400

His Met Arg Phe Ile Pro Leu Arg His Gly Lys Tyr Met Val Ser Ser

10

405

410

415

Lys Arg Trp Pro Asn Trp Phe Met Tyr Met Glu Ser Ser Ala Ser Gly

420

425

430

Tyr Ile Arg Ser Trp Glu Asn Asn Pro Gly Pro Gln Gly His Trp Ser

435

440

445

15

Ile Thr

450

20

What is claimed is:

1. A protein having following properties:

(1) having hemolytic activity;

5 (2) having a molecular weight of about 50,000 Da (determined by SDS gel electrophoresis); and

(3) having the amino acid sequence represented by any of SEQ ID NO 1 to SEQ ID NO 3 as a partial amino acid sequence.

10 2. The protein according to claim 1, wherein the protein is obtained from nematocyst of *Carybdea rastonii*.

3. A protein having the hemolytic activity which has the same amino acid sequence as the hemolytic active protein  
15 according to claim 1, or the amino acid sequence modified by the addition and deletion of one or more amino acid, and/or the substitution by other amino acid to said amino acid sequence, and which is obtained from the cultivated product of the transformed cell prepared by genetic recombinant technique.

20

4. A protein having amino acid sequence represented by SEQ ID NO 5, or the amino acid sequence modified by the addition and deletion of one or more amino acid, and/or the substitution by other amino acid to said amino acid sequence, and having  
25 hemolytic activity.

5. The protein having hemolytic activity according to claim 3 or 4, wherein said protein is obtained from cultivated solution of transformed cell prepared by genetic recombinant





11. A process for preparing a protein having hemolytic activity comprising culturing or growing a host cell as claimed in claim 10, and recovering the protein from said host cell or culture solution.

12. A pharmaceutical composition comprising the protein according to any one of claims 1 to 5 as an active component.

13. The pharmaceutical composition according to claim 12, wherein the composition has platelet agglutination effect.

14. An antibody whose antigens are protein according to any one of claims 1 to 5, or those partial peptides.

15. A pharmaceutical composition using the antibody according to claim 14.

16. A pesticide comprising the protein according to any one of claims 1 to 5 as an active component.

ABSTRACT OF THE DISCLOSURE

Novel proteins providing the new approach to development  
of the drugs and pesticides with the use or application of a  
5 hemolytic activity, and novel proteins having the following  
properties and the genes encoding thereof are provided:

(1) having hemolytic activity;

(2) having a molecular weight of about 50,000 Da  
(determined by SDS gel electrophoresis);

10 (3) having an amino acid sequence represented by any of  
SEQ ID NO 1 to SEQ ID NO 3 as a partial amino acid sequence;  
and

(4) having an amino acid sequence represented by SEQ ID  
NO 5 as the full amino acid sequence.

15

**DECLARATION AND POWER OF ATTORNEY - PATENT APPLICATION**

As a below named inventor, I hereby declare that my citizenship, postal address and residence are as stated below; that I verily believe I am the original, first and sole inventor (if only one inventor is named below) or a joint inventor (if plural inventors are named below) of the invention entitled:

**Novel Hemolytic Active Proteins and Genes Encoding The Same**

the specification of which

\_\_\_\_\_ is attached hereto, or  
x was filed on March 30, 1999 as Application Serial No. PCT/JP99/01607 and  
 was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information known to be material to patentability as defined in 37 CFR §1.56. I hereby claim foreign priority benefits under Title 35, United States Code §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)	Priority Claimed
<u>JP10-88569</u> (Number)	<u>JAPAN</u> (Country)
<u>01.04.1998</u> (Day/Month/Year)	<u>Yes</u>
_____ (Number)	_____ (Country)
_____ (Day/Month/Year)	_____

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information known to be material to patentability as defined in 37 CFR §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status)
_____	_____	_____

I hereby appoint as principal attorneys Martin Fleit, Reg. No. 16,900; Herbert I. Cantor, Reg. No. 24,392; James F. McKeown, Reg. No. 25,406; Donald D. Evenson, Reg. No. 26,160; Joseph D. Evans, Reg. No. 26,269; Gary R. Edwards, Reg. No. 31,824; Jeffrey D. Sanok, Reg. No. 32,169; Richard R. Diefendorf, Reg. No. 32,390; and Paul A. Schnose, Reg. No. 39,361, to prosecute and transact all business in the Patent and Trademark Office connected with this application and any related United States and international applications. Please direct all communications to:

**Evenson, McKeown, Edwards & Lenahan, P.L.L.C.**

**1200 G Street, N.W., Suite 700**

**Washington, D.C. 20005**

**Telephone: (202) 628-8800**

**Facsimile: (202) 628-8844**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Sept. 13, 2000

(date)

Hiroshi Nagai  
 (signature of 1st inventor)



**23911**

PATENT TRADEMARK OFFICE

DECLARATION AND POWER OF ATTORNEY

Attorney Docket No. \_\_\_\_\_

Page 2

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Sep. 25, '00

(date)

Terumi Nakajima

(signature of 2nd inventor)

**INVENTOR:**

Citizenship:

Post Office Address/

Residence:

(date)

(signature of 3rd inventor)

**INVENTOR:**

Citizenship:

Post Office Address/

Residence:

(date)

(signature of 4th inventor)

**INVENTOR:**

Citizenship:

Post Office Address/

Residence:

(date)

(signature of 5th inventor)

**INVENTOR:**

Citizenship:

Post Office Address/

Residence:

(date)

(signature of 6th inventor)